The Application of Plastic Compression to Modulate Fibrin Hydrogel Mechanical Properties

Matthew G. Haugh[a,b]*, Stephen D. Thorpe[a,b], Tatiana Vinardell[a,b], Conor T. Buckley[a,b] and Daniel J. Kelly[a,b]

[a] Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland.

[b] Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin 2, Ireland.

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* Corresponding Author

Matthew G. Haugh, B.A., B.A.I., Ph.D.

Department of Mechanical and Biomedical Engineering,

College of Engineering and Informatics,

National University of Ireland Galway,

Galway, Ireland.

Email: matthew.haugh@nuigalway.ie

Abstract

The inherent biocompatibility of fibrin hydrogels makes them an attractive material for use in a wide range of tissue engineering applications. Despite this, their relatively low stiffness and high compliance limits their potential for certain orthopaedic applications. Enhanced mechanical properties are desirable so as to withstand surgical handling and *in vivo* loading after implantation and additionally, can provide important cues to cells seeded within the hydrogel. Standard methods used to enhance the mechanical properties of biological scaffolds such as chemical or thermal crosslinking cannot be used with fibrin hydrogels as cell seeding and gel formation occurs simultaneously. The objective of this study was to investigate the use of plastic compression as a means to improve the mechanical properties of chondrocyte-seeded fibrin hydrogels and to determine the influence of such compression on cell viability within these constructs. It was found that the application of 80% strain to fibrin hydrogels for 30 minutes (which resulted in a permanent strain of 47.4%) produced a 2.1-fold increase in the subsequent compressive modulus. Additionally, chondrocyte viability was maintained in the plastically compressed gels with significant cellular proliferation and extracellular matrix accumulation observed over 28 days of culture. In conclusion, plastic compression can be used to modulate the density and mechanical properties of cell-seeded fibrin hydrogels and represents a useful tool for both in theatre and *in vitro* tissue engineering applications.

1. Introduction

Fibrin is a polymeric protein that acts with platelets to form a thrombus or blood clot and as such constitutes an intrinsic scaffold for repair and regeneration (Orlando *et al.*, 2012). Accordingly, fibrin hydrogels are an attractive material for use in tissue engineering; possessing inherent biocompatibility, biodegradability and suitable interaction with cells and other macromolecules (Sierra, 1993, Bensaïd *et al.*, 2003, Ahmed *et al.*, 2008, Breen *et al.*, 2009). As such fibrin has been used as a scaffold to deliver cells, drugs and therapeutic molecules in a wide range of tissue engineering applications (Albes *et al.*, 1994, Eyrich *et al.*, 2007, Willerth *et al.*, 2007, Pelaez *et al.*, 2009, Bhang *et al.*, 2007b, Ahearne *et al.*, 2011, Hyatt *et al.*, 2010, Steward *et al.*, 2012, Deponti *et al.*, 2012, O'Heireamhoin *et al.*, 2012, Thorpe *et al.*, 2012). However, despite these qualities, the relatively weak mechanical properties of fibrin hydrogels may limit their use in orthopaedic applications (Hokugo *et al.*, 2006, Mol *et al.*, 2005, Jockenhoevel *et al.*, 2001). The mechanical properties of scaffolds used in tissue engineering are important for establishing appropriate mechanical support to cells within load bearing defects and facilitating manipulation of these constructs prior to implantation (Rosso *et al.*, 2005). Additionally, several studies have shown that the stiffness of a substrate can direct cellular activity such as attachment, migration and differentiation (Engler *et al.*, 2004, Engler *et al.*, 2006, Yeung *et al.*, 2005, Haugh *et al.*, 2011b, Murphy *et al.*, 2012). Thus mechanical properties take on further importance.

The mechanical weakness of fibrin hydrogels arises from the highly hydrated nature of these gels (i.e. the low protein to water ratio). Crosslinking techniques are commonly used to improve the properties of protein based biomaterials, however these techniques

can leave behind cytotoxic residues (Haugh *et al.*, 2011b, Weadock *et al.*, 1983). Additionally as gelation and cell seeding typically take place concurrently during fabrication of hydrogels, the use of cytotoxic crosslinking reagents would not be viable. Recently it has been shown that dense collagen hydrogels can be produced through unconfined plastic compression (PC), which results in significant exclusion of water from the hydrogels and a large increase in strength (Brown *et al.*, 2005, Abou Neel *et al.*, 2006, Bitar *et al.*, 2007). The uncomplicated nature of this process, coupled with its ability to rapidly improve mechanical properties while having no substantial effect on cell viability within the gels, also make it practical for incorporation in cell delivery techniques in theatre (Brown *et al.*, 2005). The results of these studies suggest that plastic compression represents a promising technique for improving the properties of fibrin hydrogels due to their structural similarities to collagen-based hydrogels.

Despite the widespread use of fibrin hydrogels, their mechanical properties restrict their application in tissue engineering. Plastic compression has been shown to improve the properties of collagen gels but its effects on fibrin-based hydrogels have yet to be evaluated. Therefore, the overall goal of this study was to investigate the feasibility of improving the mechanical properties of fibrin gels through the use of plastic compression. The specific objectives were to determine 1) the increase in stiffness of fibrin hydrogels following plastic compression, 2) the stability of this increase over time and 3) the effect of plastic compression on chondrocyte viability and cartilaginous matrix production within the hydrogels.

2. Materials and Methods

2.1 Cell Isolation and Preparation of Fibrin Gel Constructs

The effects of plastic compression on cell viability were assessed using porcine chondrocytes. Full-depth slices of cartilage were harvested under aseptic conditions from the femoral condyles of 3 four month old porcine donors (~50 kg), and rinsed thoroughly with PBS containing 200 U/mL penicillin, 200 µg/mL streptomycin (GIBCO, Biosciences, Dublin, Ireland). Chondrocytes were isolated from cartilage slices through incubation with DMEM/F12 containing collagenase type II (0.5 mg/mL) (all from Sigma–Aldrich, Arklow, Ireland) for 18 h under constant rotation at 37°C. The cell suspension was passed through a 40 µm pore-size cell sieve (BD Falcon, Unitech, Ireland) and the filtrate centrifuged and rinsed with PBS twice. Cells were seeded at a density of $5x10^4$ cells/cm^2 in 175 cm^2 T-flasks (Sarstedt, Neumbrecht, Germany) and expanded (1:2 ratio) to passage one (P1). Cell number and viability were determined using a haemacytometer and 0.4% trypan blue staining. Isolated chondrocytes from all joints were pooled and maintained in DMEM/F-12 (Sigma–Aldrich, Dublin, Ireland) supplemented with 10% v/v fetal bovine serum (FBS) and 100 U/mL penicillin, 100 µg/mL streptomycin (GIBCO, Biosciences, Dublin, Ireland) during the expansion phase.

Following isolation and expansion to P1, chondrocytes from the three donors were pooled, then suspended at $7.5x10^6$ cells/mL in a 40 mg/mL solution of fibrinogen in DMEM/F-12 (Sigma-Aldrich, Arklow, Ireland). In order to fabricate the gels, 10 U/mL thrombin (0.5 U thrombin per mg of fibrinogen; Sigma-Aldrich, Arklow, Ireland) was added to the fibrinogen/cell suspension (Zhao *et al.*, 2008). The resulting solution was

mixed by pipette and cast in a stainless steel mould and left to clot at 37°C for 10 min to produce 3 mm thick cylindrical constructs (Ø5 mm, $4.4x10^5$ cells). Fibrin gel constructs were maintained in DMEM/F-12 (Sigma–Aldrich, Arklow, Ireland) supplemented with 10% v/v fetal bovine serum (FBS) and 100 U/mL penicillin, 100 µg/mL streptomycin (GIBCO, Biosciences, Dublin, Ireland), 1 mg/mL insulin and 50 mg/mL ascorbic acid (Sigma–Aldrich, Arklow, Ireland). Aprotinin (Sigma-Aldrich, Arklow, Ireland) was also added to the media at a concentration of 0.0875 IU/mL to prevent degradation of the fibrin constructs. Medium was changed every 3-4 days.

2.2 Plastic compression

Fibrin gel constructs were compressed using a bioreactor system developed in our laboratory as previously described (Thorpe *et al.*, 2010, Haugh *et al.*, 2011a). Unconfined compression was applied using an impermeable platen to 80% strain; this deformation was then maintained for 30 min in a humidified atmosphere at 37°C and 5% CO_2. After the application of plastic compression the constructs were placed in 6 well plates containing supplemented CDM and maintained for 28 days in standard cell culture conditions.

2.3 Mechanical Testing

Unconfined compression testing with impermeable, un-lubricated platens was used to determine the effect of plastic compression on the modulus of fibrin gels. Mechanical testing of the samples was carried out using a mechanical testing machine (Z050, Zwick/Roell, Germany) fitted with a 5 N S-type load cell and all testing was carried out in a bath of PBS. The accuracy of this system was as follows: crosshead speed had 0.05

% accuracy of the set speed and the drive systems had a travel resolution of 0.0828 μm with positioning resolution accuracy ± 2 μm. The S-type load cell used in this study was calibrated in tension and compression with a 5 N load capacity and the force readings were accurate to within 0.06 % of resultant force. Testing was conducted at a strain rate of 10%/min and the modulus was defined as the slope of a linear fit to the stress-strain curve over 2-5% strain (Haugh *et al.*, 2009, Haugh *et al.*, 2011b). The height of the samples was calculated using a preload threshold (5 mN) to establish contact between the sample and the upper platen before initiation of the compression tests, this data was used to calculate the level of plastic compression at days 0 and 28.

2.4 Quantitative Biochemical Analysis

Samples were harvested at days 0 and 28. At each time point the samples were weighed and their dimensions measured before being frozen (-85°C) for analysis at a later date. Prior to analysis, samples were thawed and digested with 125 μg/mL papain in 0.1 M sodium acetate, 5 mM $_{L}$-cysteine HCL, 0.05 M EDTA (all Sigma-Aldrich, Arklow, Ireland), pH 6.0 at 60°C under constant rotation for 18 hr. Cell number was evaluated using the Hoechst 33258 DNA assay, which fluorescently labels double-stranded DNA (Sigma-Aldrich, Germany), according to a previously published protocol and using calf thymus DNA (Sigma-Aldrich, Arklow, Ireland) as a standard (Kim *et al.*, 1988). Sulphated glycosaminoglycan (sGAG) content was measured using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland) using shark derived chondroitin sulphate (Sigma-Aldrich, Arklow, Ireland) as a standard. All assays were carried out with a minimum of three samples analysed in triplicate for each group at every time point.

2.5 Confocal Microscopy

Cell viability within the compressed and control samples was assessed using a LIVE/DEAD assay kit (Invitrogen, Biosciences, Dublin, Ireland) at 1 and 28 days. Fibrin gel constructs were first washed in PBS and then placed in a calcein acetoxymethyl and ethidium homodimer-1 solution for 1 hour to stain live and dead cells. The samples were then rinsed three times in PBS and images of the constructs were captured using a confocal microscope (LSM510 META, Carl Zeiss, Germany). Confocal scans were taken as close to the centre of the hydrogel as it was possible to focus (approx. 100 μm from the surface).

2.6 Statistical Analysis

Results are expressed as mean ± standard deviation. Two-way analysis of variance (ANOVA), with time and gel type (compressed/uncompressed) as the independent factors, followed by pairwise multiple comparison procedures (Holm-Sidak test) was used depending on the number of variables to evaluate the results from the quantitative biochemical assays. Statistical significance was declared at $p \leq 0.05$.

3. Results

3.1 The effect of plastic compression on construct dimensions and mechanical properties

At day 0 it can be seen that 80% compression resulted in a plastic (or permanent) deformation of 47.4% such that constructs were now 52.6% of their original thickness. By day 28 the samples had recovered (or had swollen to) to 69.8% of their original

thickness (or 30.2% plastic strain, Fig. 1A). The compressive modulus of the plastically compressed gels was significantly greater than the control group at both time points and the modulus of both groups increased over the 28 day incubation period (Fig. 1B, Table 1). At day 0 plastic compression resulted in a 2.12-fold increase in compressive modulus over uncompressed gels (5.95 vs. 12.63 kPa; $p<0.05$). Despite the change in deformation and elaboration of matrix (see below), plastic deformation still had a beneficial effect on construct properties in the long-term, with these constructs still 1.44 times stiffer than the uncompressed gels after 28 days of culture (24.68 vs. 35.74 kPa; $p<0.05$, Table 1).

3.2 The effect of plastic compression on cell viability and matrix deposition

Live dead staining was used to asses cell viability in both groups at day 0 and 28 (Fig. 2). No significant differences in cell viability can be seen between the uncompressed and compressed at either time point. Additionally a change in cell morphology was observed in both groups; at day 0 the cells appear rounded within the gels, while by day 28 cells had adopted a spread morphology consistent with cells that have attached to the surrounding matrix.

Cell number, as measured by DNA content, can be seen to increase significantly over the 28 day incubation period in both groups (Fig. 3A, $p<0.05$). By day 28, DNA content is significantly higher in the uncompressed constructs; however DNA content in the plastically compressed group still increases from day 0 to 28 indicating significant cell proliferation within the constructs. The sGAG content within the gels increased significantly over time in both groups (Fig. 3B&C, $p<0.001$). Total sGAG content was

significantly greater in the uncompressed group, however when sGAG content was normalised to DNA there was no significant difference between compressed and uncompressed constructs.

Discussion

Plastic compression has been previously shown to increase the compressive properties of cell seeded collagen gels (Brown *et al.*, 2005, Abou Neel *et al.*, 2006, Bitar *et al.*, 2007). This method is both effective and uncomplicated in its application; however its potential to enhance the properties of fibrin hydrogels has not been explored. With this in mind, we sought to investigate the feasibility of enhancing the mechanical properties of fibrin gels through the use of plastic compression and to determine its effect on the viability of cells seeded within them. We found that the application of plastic compression to fibrin hydrogels produced an initial 2.1-fold increase in compressive modulus. A significant difference in modulus between uncompressed and plastically compressed gels was maintained over 28 days of culture with compressed gels still 1.4 stiffer than the uncompressed gels at this time point. Additionally, plastic deformation and cellular viability was maintained in the plastically compressed gels with significant cellular proliferation and extracellular matrix accumulation over 28 days. Taken together, these results indicate that the application of plastic compression to fibrin hydrogels represents a useful tool for both in theatre and *in vitro* tissue engineering applications.

In this study, we investigated the effects of plastic compression on both the mechanical properties of fibrin hydrogels and the viability of cells within them. The

effect of altering fibrinogen solution concentration prior to gelation on the strength and durability of cell seeded fibrin hydrogels has been extensively investigated in previous studies (Jeon *et al.*, 2005, Bhang *et al.*, 2007a, Zhao *et al.*, 2008). However, this approach has limitations owing to the solubility of fibrinogen. Therefore in this study, we sought to use plastic compression to effectively increase gel density post gelation, bypassing limitations due to solubility. The starting concentration of fibrinogen used in this study was chosen to be relevant for surgical applications as it is similar to the concentrations of commercially available surgical fibrin sealants (Hyatt *et al.*, 2010). Higher fibrinogen concentrations have been used previously in the literature (Hyatt *et al.*, 2010, Jeon *et al.*, 2005), however we have found that it is increasingly difficult to get fibrinogen into solution at higher concentrations, which in turn makes it more difficult to sterile filter the solution and to produce a homogenous construct post-gelation. Therefore, we sought to investigate the effect of plastic compression of fibrin hydrogels using a fibrinogen solution concentration which was both similar to products approved for surgical use and that facilitated the fabrication of a sterile homogeneous gel. In this study the effect of plastic compression on mechanical properties and cell viability was investigated at 0 and 28 day time points. These time points were chosen as they allowed the initial effects of plastic compression to be evaluated in addition to the stability of the induced changes to be determined over an extended culture period. The disadvantage of this approach is that it provides no information on changes in deformation and cellular activity between days 0 and 28. However, given that plastic compression did not appear to have a significant impact on the viability of the cells within the fibrin hydrogels over the 28 day time period, the time points used in this study were sufficient to address the aims of the study.

Mechanical testing showed that plastic compression resulted in an initial 2.14 fold increase in compressive modulus which was reduced to a 1.4 fold increase after 28 days of culture (Fig. 1). The initial effect of plastic compression on construct mechanical properties is important in applications such as articular cartilage regeneration, where the gels will be loaded shortly after implantation. While plastic compression has been used previously to enhance the properties of collagen gels, its effects on compressive modulus were not investigated, making a comparison to our results difficult (Brown *et al.*, 2005, Abou Neel *et al.*, 2006, Bitar *et al.*, 2007). Additionally, there are differences in the gelation mechanisms between collagen and fibrin hydrogels. Fibrin hydrogels are formed when fibrinogen is crosslinked enzymatically by thrombin, while collagen gels self-assemble to form a network of entangled but non-covalently linked fibrils (Breen *et al.*, 2009, Abou Neel *et al.*, 2006). In both cases any increase in mechanical properties is the result of an increase in protein density as fluid is driven out of the gels during plastic compression.

The temporal increase in sGAG content within cell seeded fibrin hydrogels found in this study is comparable to previous studies (Eyrich *et al.*, 2007). We found a sGAG content in the range of 112-152 ng/construct at 28 days using porcine chondrocytes ($4.4x10^5$ cells), while Eyrich *et al*. (2007) found a sGAG content of approximately 250 ng/construct at 35 days using bovine chondrocytes ($5x10^5$ cells). This variation in sGAG content is within a range that may be accounted for by the difference in the species of cell source, time point of analysis and construct dimensions used in the two studies. Using chondrocytes seeded onto polylactide/fibrin gel

composites, Zhao *et al.* (2009) reported an sGAG content of approximately 1.7 mg/cm^3 at day 21, which compares well to our value of 2.7 and 2.8 mg/cm^3 at 28 days for uncompressed and compressed fibrin gels respectively (Table 1, Zhao *et al.* 2009). While levels of sGAG synthesis were comparable in compressed and uncompressed constructs, chondrocytes proliferation was lower in compressed hydrogels. Previous studies have reported that cellular proliferation within fibrin hydrogels decreases with increases in fibrinogen concentration (Cox *et al.*, 2004, Ho *et al.*, 2006, Duong *et al.*, 2009, des Rieux *et al.*, 2009), which may be due to reduced permeability in these higher fibrinogen concentration constructs (Chiu *et al.*, 2012). A similar mechanism may explain the lower levels of chondrocyte proliferation in the compressed hydrogels.

The results of this study show that plastic compression can be used to produce a stable increase in the fibrin concentration of fibrin hydrogels resulting in a significant increase in mechanical properties. The improvement in mechanical properties achieved through plastic compression increases the suitability of fibrin hydrogels for 'in theatre' applications and alternatively could also reduce the length of *in vitro* culture needed to attain load-bearing properties prior to implantation. Additionally, while there was a significant difference in cell number between the compressed and uncompressed groups at 28 days, the substantial increases in cell number and sGAG content observed within the compressed hydrogels over the 28 day culture period demonstrates that plastic compression had negligible effects on cellular viability and biosynthesis within the fibrin gels. Previous studies have shown that the release of therapeutic molecules from fibrin gels is enhanced by increasing the fibrin content of the hydrogels (Jeon *et al.*, 2005, Bhang *et al.*, 2007a), thus plastic compression may have further benefits not

explored in this study. It should also be noted that fibrin gels typically degrade *in vitro* over the time frames used in this study. In order to prevent this degradation we added a protease inhibitor, Aprotinin, to the culture media. Previous work has also found that increasing the fibrin concentration (≈50 mg/mL) slows the degradation of the constructs *in vitro* (Eyrich *et al.*, 2007). Thus it is possible that in addition to improved mechanical properties, the plastically compressed fibrin gels used in this study (≈84 mg/mL at day 0) may also degrade slower.

Conclusions

Plastic compression resulted in a significant increase in the compressive properties of fibrin gels without impairing cellular viability and subsequent extracellular matrix synthesis. The application of plastic compression to fibrin gels may provide a useful tool in tissue engineering applications that require an increase in gel density and mechanical properties immediately prior to implantation or at the onset of *in vitro* culture.

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Address correspondence and reprints requests to:

Matthew G. Haugh, B.A., B.A.I., Ph.D.

Department of Mechanical and Biomedical Engineering,

College of Engineering and Informatics,

National University of Ireland Galway,

Galway, Ireland.

Phone: +353-91-524411

Fax: +353-91-563991

Email: matthew.haugh@nuigalway.ie

Figure Captions:

Figure 1. Plastic compression results in a stable increase in compressive modulus. (A) Plastic (permanent) strain of compressed gels at days 0 and 28. (B) Compressive modulus of uncompressed and compressed fibrin constructs at days 0 and 28. $^{A}p<0.05$ vs. uncompressed at the corresponding time-point. $^{B}p<0.05$ vs. uncompressed at day 0. $^{C}p<0.05$ vs. compressed at day 0.

Figure 2. Cell viability is not compromised by plastic compression. Images show representative sections of live (green) and dead (red) cells within the middle of uncompressed and compressed fibrin constructs at days 0 and 28.

Figure 3. Significant levels of proliferation and ECM synthesis were found within uncompressed and compressed fibrin constructs. (A) Total DNA content per construct, (B) sGAG content per construct and (C) sGAG content normalised to DNA content. $^{A}p<0.05$ vs. uncompressed at the corresponding time-point. $^{B}p<0.05$ vs. uncompressed at day 0. $^{C}p<0.05$ vs. compressed at day 0.

Table 1. Fibrin gel properties.

Figure 1

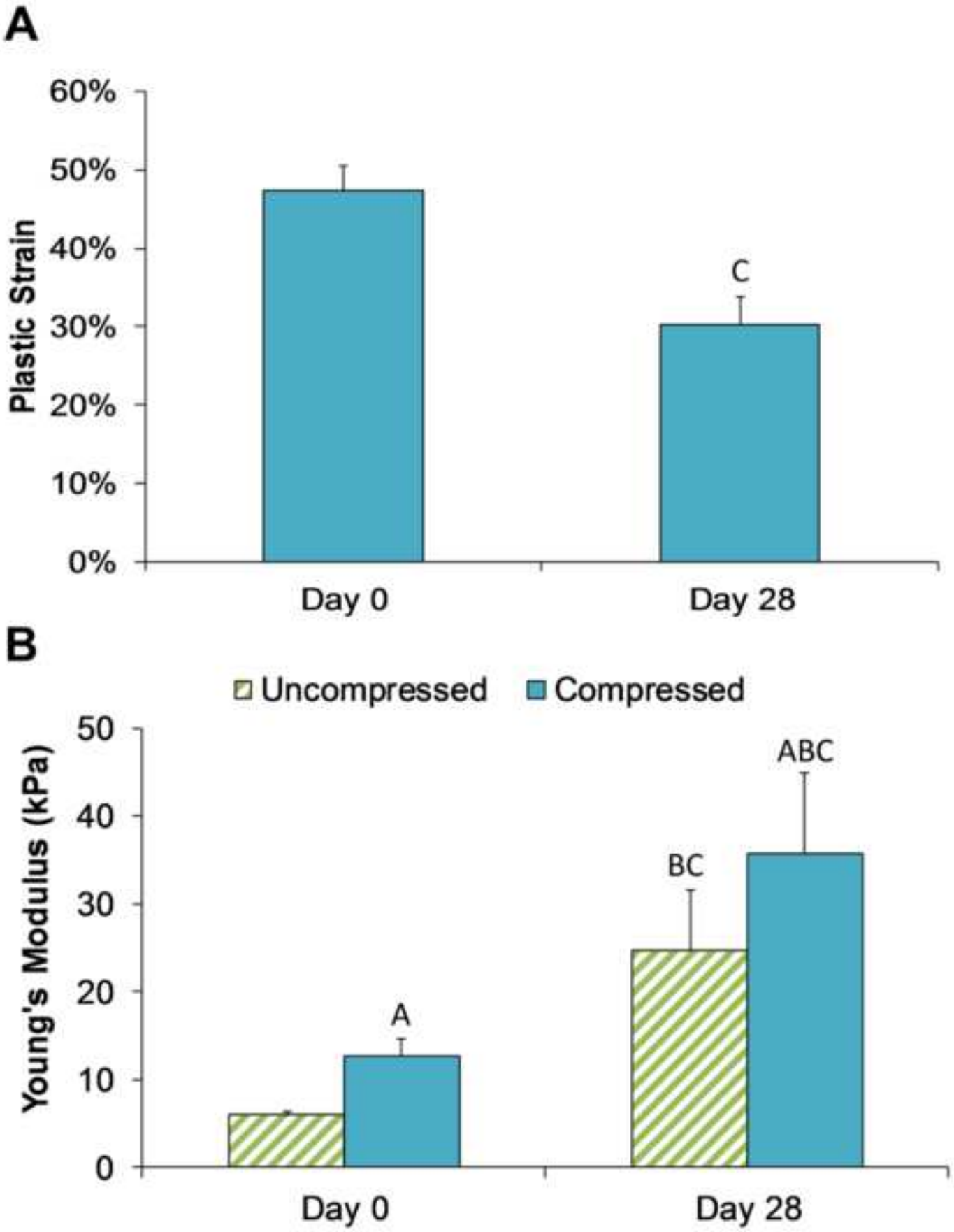

A
Plastic Strain
60%
50%
40%
30%
20%
10%
0%
C
Day 0
Day 28
B
Uncompressed
Compressed
Young's Modulus (kPa)
50
40
30
20
10
0
A
BC
ABC
Day 0
Day 28

Figure 2

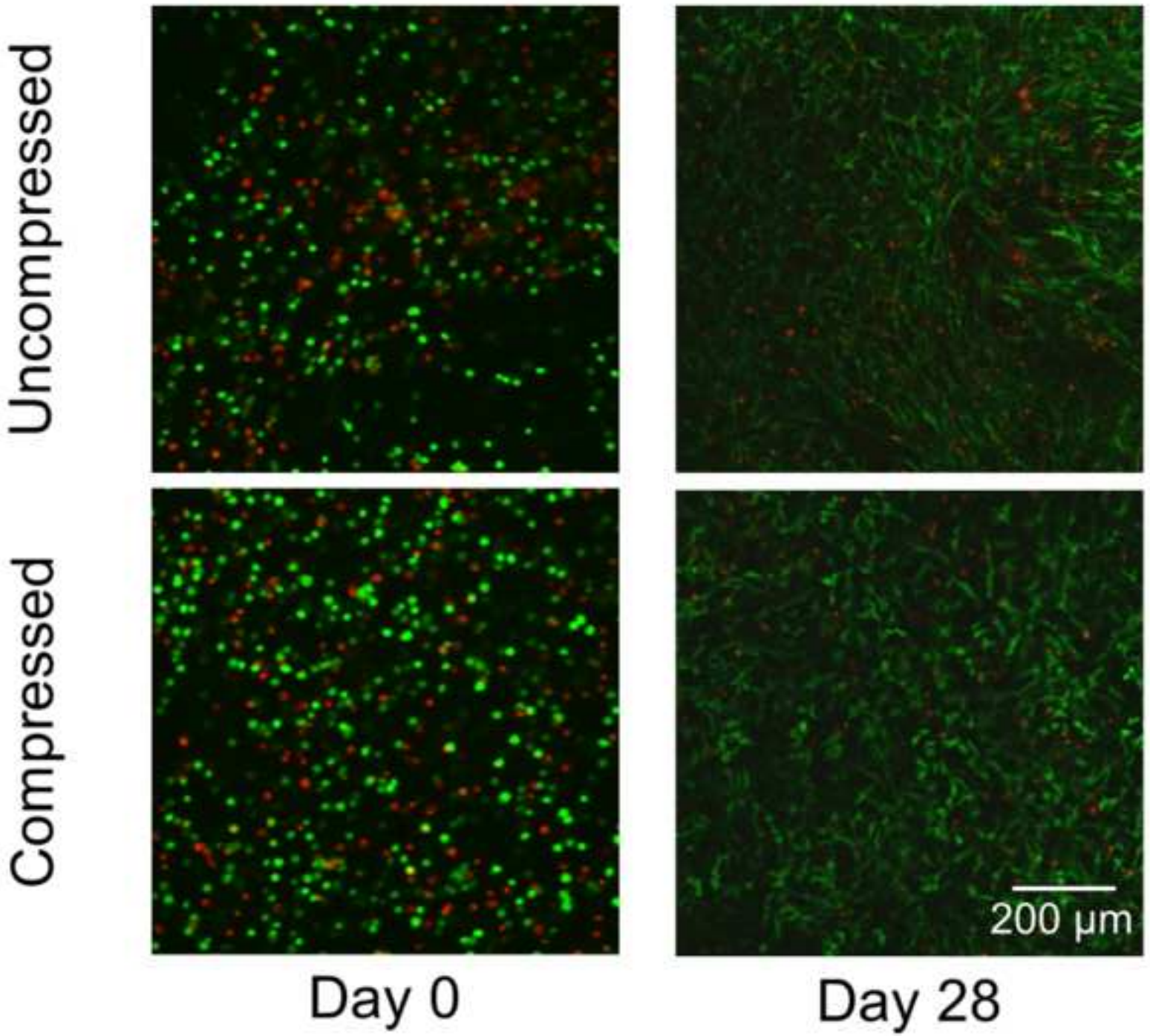
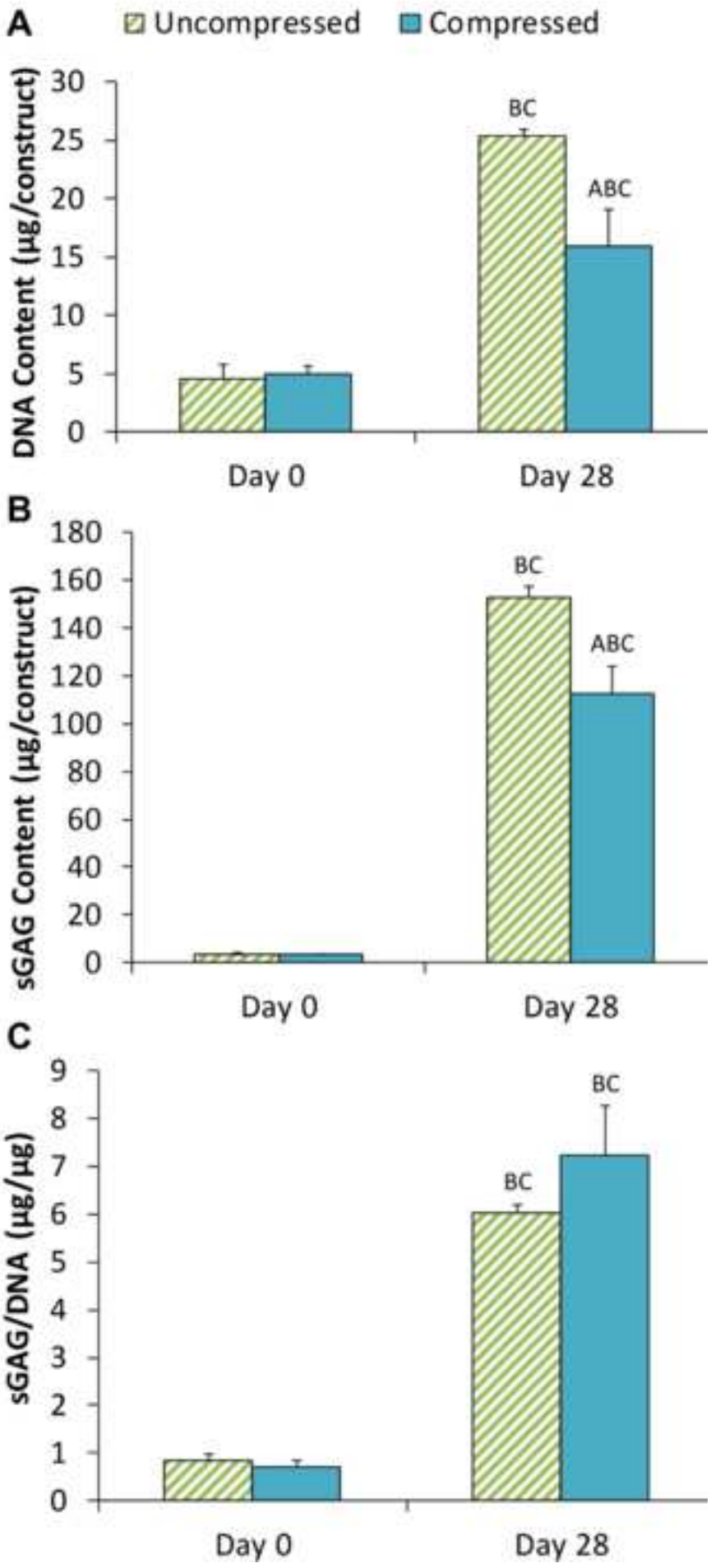


Figure 3


A
Uncompressed
Compressed
DNA Content (µg/construct)
30
25
20
15
10
5
0
BC
ABC
Day 0
Day 28
B
sGAG Content (µg/construct)
180
160
140
120
100
80
60
40
20
0
BC
ABC
Day 0
Day 28
C
sGAG/DNA (µg/µg)
9
8
7
6
5
4
3
2
1
0
BC
BC
Day 0
Day 28

Table

Group	Uncompressed		Compressed	
	Day 0	Day 28	Day 0	Day 28
Mass (mg)	57.55	55.73	37.71	43.05
Height (mm)	2.98	3.16	1.57	2.08
Ø (mm)	4.88	4.77	5.40	4.97
Modulus (kPa)	5.95	24.68	12.63	35.74
DNA (µg)	4.52	25.28	4.96	15.90
sGAG (µg)	3.70	152.53	3.44	112.78
sGAG (mg/cm^3)	0.07	2.70	0.10	2.83